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Characterization and Solubilization of the FMRFamide Receptor of Squid

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Abstract. The optic lobe of squid (*Loligo pealei*) contains FMRFamide receptors that can bind an iodinated FMRFamide analog: [^{125}I]-desaminoTyr-Phe-norLeu-Arg-Phe-amide ([^{125}I]-daYFnLRFa). Radioligand binding assays revealed that squid FMRFamide receptors are specific, saturable, high affinity sites (K_d = 0.15 n*M*) densely concentrated in optic lobe membranes (B_{max} = 237 fmole/mg protein). The receptors appeared to be coupled to G_s because guanine nucleotides inhibit receptor binding and the stimulation of adenylate cyclase by FMRFamide is GTP-dependent. Both the binding and cyclase data showed that FMRFamide, but not FMRF-OH, interacts at FMRFamide receptors; thus the C-terminal Arg-Phe-amide is critical for binding. The high binding affinity of FMRFamide (0.4 n*M* IC_{50}) was specific for FMRFamide-like peptides. The structure-activity relations of many FMRFamide analogs were defined in detail and were nearly identical for both the membrane-bound and detergent-solubilized receptors. We also found that squid optic lobe contains FMRFamide-like reactivity as measured with both a radioimmunoassay and a radioreceptor assay. Moreover, we have sequenced a fragment of genomic DNA that encodes a FMRFamide precursor. Our findings in sum suggest that FMRFamide is a neurotransmitter in squid optic lobe, and that this tissue is a good source from which to purify FMRFamide receptors.

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Abbreviations: In addition to standard one- and three-letter abbreviations for amino acids, the following are used: the letter "a" at *C*-terminal ends of peptides signifies an amide; ac, acetyl; BSA, bovine serum albumin fraction V; cAMP, adenosine-3′,5′-cyclic monophosphate; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonic acid; CHAPSO, 3-[(3-cholamido-propyl)-dimethylammonio]-2-hydroxy-1-propane sulfonic acid; daY, desaminotyrosine; dNTPs, equimolar mixture of dATP, dGTP, dCTP, and dTTP; DTT, dithiothreitol; EDTA, disodium ethylenediamine tetraacetate; FMRFamide, Phe-Met-Arg-Phe-NH_2; Gpp[NH]p, guanylyl-imidodiphosphate; GTP[γ]S, guanosine-5′-*O*-(3-thiotriphosphate); HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid; IBMX, isobutylmethylxanthine; MES, 2-[*N*-morpholino]ethane sulfonic acid; PIPES, piperazine-*N*,*N*′-bis-2-ethane sulfonic acid; nL, norleucine; NS, nonspecific binding; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonylfluoride; RIA, radioimmunoassay; SB, specific binding; SQM, squid optic lobe membranes; TB, total binding; TIC, L-1,2,3,4 tetrahydroisoquinoline-3-carboxylic acid.

Introduction

The tetrapeptide FMRFamide (Phe-Met-Arg-Phe-NH_2) functions as a neurotransmitter in many invertebrates. Biochemical and molecular biological studies indicate that FMRFamide-related peptides constitute a large and diverse transphyletic peptide family (Price and Greenberg, 1989). A single species often contains more than one FMRFamide-related peptide; multiple FMRFamide-related peptides are encoded by a single mRNA in *Aplysia* (Taussig and Scheller, 1986), *Drosophila* (Schneider and Taghert, 1988), *Lymnaea* (Linacre *et al.*, 1990), and *Caenorhabditis* (Rosoff *et al.*, 1992). Acting through G-protein-regulated pathways, FMRFamide activates K^+ channels in *Aplysia* sensory neurons (Abrams *et al.*, 1984) and inhibits Ca^{2+} channels in *Helisoma* neurons (Man-Son-Hing *et al.*, 1989). The peptide also excites the heart in *Macrocallista* (Price and Greenberg, 1977), *Hirudo* (Kuhlman *et al.*, 1985), *Helix* (Payza, 1987), *Octopus* (Martin and Voigt, 1987; Voigt *et al.*, 1981), and *Sepia* (Kling and Jakobs, 1987). In addition to their diverse effects in invertebrates, FMRFamide-related peptides modulate opioid analgesia, tolerance, and dependence in rats

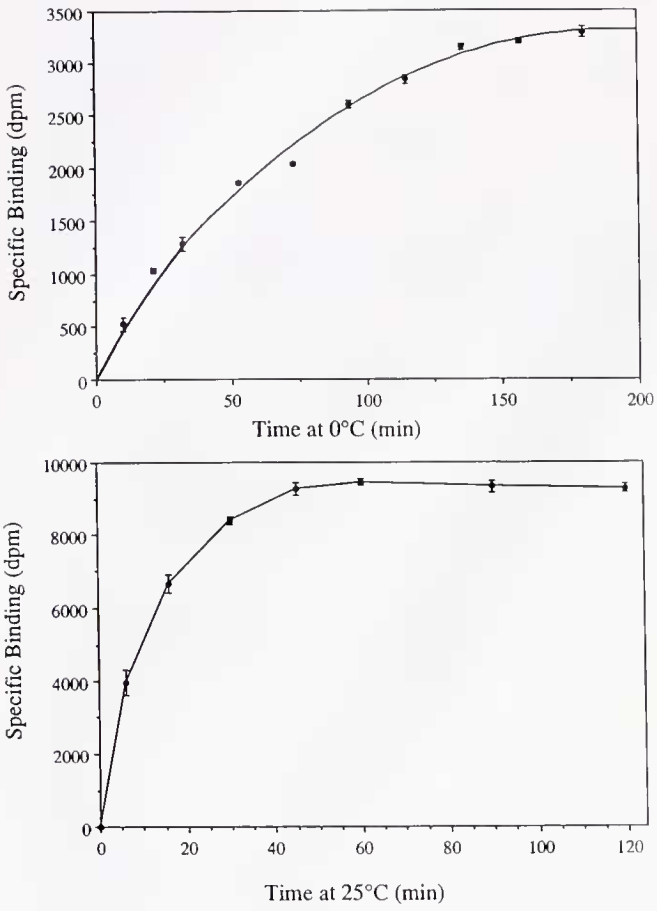


Figure 1. Time course of FMRFamide receptor binding in SQM. For the upper and lower figures respectively, [^{125}I]-daYFnLRFa (0.026 and 0.032 n*M*) was incubated with SQM (50 and 10 μg protein) at 0° and 25°C. The total and nonspecific binding were determined in triplicate in single experiments at the times indicated, and the specific binding (± SEM) is plotted against time.

(Lake *et al.*, 1991; Malin *et al.*, 1990a, b; Malin *et al.*, 1993; Tang *et al.*, 1984; Yang *et al.*, 1985); for reviews, see Raffa (1988) and Rothman (1992). The actions of FMRFamide and the mammalian FMRFamide-related peptide Neuropeptide FF (NPFF) appear to be mediated through binding to NPFF receptors in rats (Allard *et al.*, 1989; Payza *et al.*, 1993).

Despite numerous studies of the cellular effects of FMRFamide and related peptides, little is known about the receptors for these ligands. An *in vitro* radioligand binding assay was developed and used to characterize the receptor in *Helix* nervous tissue and heart (Payza, 1987; Payza *et al.*, 1989). These studies indicated that [^{125}I]-daYFnLRFa binds in a reversible, saturable, and specific manner to FMRFamide receptors of high (13 n*M*) and low (250 n*M*) affinity in *Helix* brain. This approach has been extended to include characterization of the rat spinal cord receptor for NPFF (Allard *et al.*, 1989; Payza and Yang, 1993). Nevertheless, purified FMRFamide receptor protein and sequence information are necessary to develop specific molecular probes with which to study receptor expression, distribution, and diversity. Thus, we sought to identify a source of molluscan nervous tissue in which FMRFamide receptors could be studied, and from which the receptors could be solubilized and eventually purified.

Here we describe the characterization, effector-coupling, and solubilization of FMRFamide receptors in the squid *Loligo pealei*. We also present evidence that FMRFamide is present in this species. *Loligo pealei* is used as our molluscan model system because squid nervous tissue is abundant and readily available, and because the relevance of FMRFamide in *Loligo* was demonstrated by its potentiation of transmitter release at the giant synapse (Cottrell *et al.*, 1989).

Materials and Methods

Materials

Frozen squid optic lobes were from Calamari, Inc. (Woods Hole, MA). The peptides FMRFa and acFnLRFa

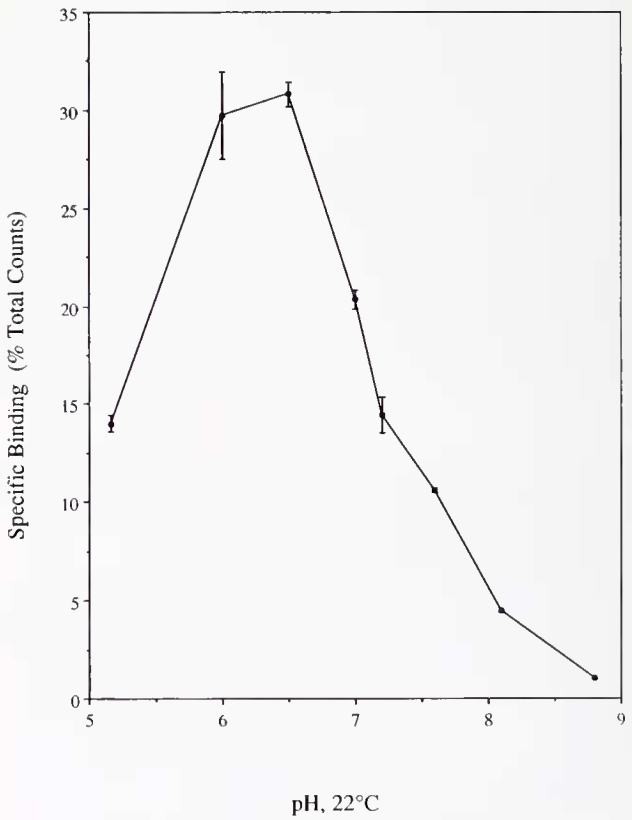


Figure 2. Dependence of FMRFamide receptor binding on pH. Squid optic lobe membranes were incubated with 0.014 n*M* [^{125}I]-daYFnLRFa in 80 m*M* Hepes/Tris buffered at the pH values indicated. After 2.5 h at 0°C, the total and nonspecific binding were determined in triplicate. The specific binding values were normalized to the total radioligand, and have been plotted against pH. Points are mean values (± SEM) from one experiment.

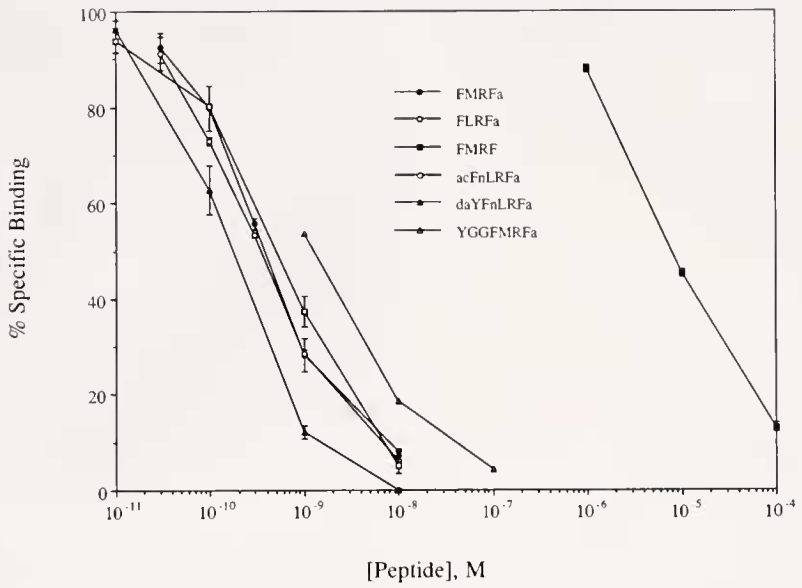


Figure 3. Specificity of FMRFamide receptor binding. The specific binding of 0.02 n*M* [^{125}I]-daYFnLRFa to SQM (50 μg protein) was determined after incubation for 2.5 h at 0°C in the presence of competing peptides at the concentrations indicated. The percentage of control specific binding has been plotted against peptide concentration. Points are means (± SEM) of triplicate determinations from single experiments, except for YGGFMRFa (duplicates from one experiment).

were from Sigma (St. Louis, MO). The YFMRFa was from Peninsula (Belmont, CA), and FnLRFa was from Cambridge Research Biochemicals (Cambridgeshire, England). The [D-F]MRFa, F[D-M]RFa, FM[D-R]Fa, and FMR[D-F]a were from Bachem (Torrance, CA). Except for daYFnLR[TIC]amide (which we made), the remaining peptides were synthesized by Dr. B. M. Dunn (University of Florida, Gainesville, FL), purified by reverse-phase HPLC, and quantitated by amino acid analysis. Aprotinin, creatine kinase, and nucleotides (Li_4)GTP[γ]S, (Li_2)GTP, (Li_2)GDP, and (Na_2)ATP were from Boehringer Mannheim (Indianapolis, IN). The (Li_4)Gpp[NH]p and (Na_2)GMP, isobutylmethylxanthine (IBMX), and digitonin were from Fluka (Ronkonkoma, NY). The CHAPS and CHAPSO were from Calbio-chem (La Jolla, CA). Bestatin was from Cambridge Research Biochemicals (Wilmington, DE).

Preparation of squid optic lobe membranes (SQM)

Squid optic lobes (12 per preparation, each ~0.15 g wet weight) were thawed and homogenized with 12 strokes of a motor-driven Potter-Elvehjem pestle in 24 ml homogenization buffer (50 m*M* Hepes-NaOH, pH 7.4, 150 m*M* NaCl, 5 mM 2-mercaptoethanol, 3 m*M* EDTA, 1 m*M* EGTA) containing 0.4 m*M* PMSF and 5 m*M* benzamidine. The homogenate was centrifuged at 3000 rpm (1,000 × *g*) for 10 min, and the pellet was rehomogenized in 24 ml homogenization buffer with inhibitors and recentrifuged. The two supernatants were combined, diluted to 200 ml with homogenization buffer, and centrifuged at 50,000 rpm (200,000 × *g*) for 30 min. This supernatant was discarded. The pellets were twice suspended in 200 ml of 20 mM Pipes-Tris (pH 6.5) and centrifuged. The final pellets were suspended in Pipes-Tris buffer to a protein concentration of 5–15 mg/ml, quick-frozen in dry ice, and stored at −20°C. Protein was measured as previously described (Chin and Goldman, 1992). These membranes retained most of their binding activity after freezing and thawing repeatedly.

Binding of [^{125}I]-daYFnLRFa to SQM

Squid optic lobe membranes (10–75 μg protein) were combined with the indicated concentrations (see figure legends) of mono-iodo [^{125}I]-daYFnLRFa, prepared and purified to 2200 Ci/mmole as before (Payza, 1987), in a total volume of 300 μl of 40 m*M* Pipes-Tris (pH 6.5), 1% BSA. Nonspecific binding was determined in the presence of 1–10 μM FMRFamide or acFnLRFa. Effects of test substances on radioligand binding were determined by including other peptides or nucleotides in the assay. The mixtures were incubated for the times and temperatures specified in the figure and table legends. The samples were then filtered through wet Whatman GF/B filters on a vacuum manifold, and the filters were washed four times with 4 ml of ice-cold Pipes buffer with 0.75% BSA. Radioactivity trapped on the filters was measured with a

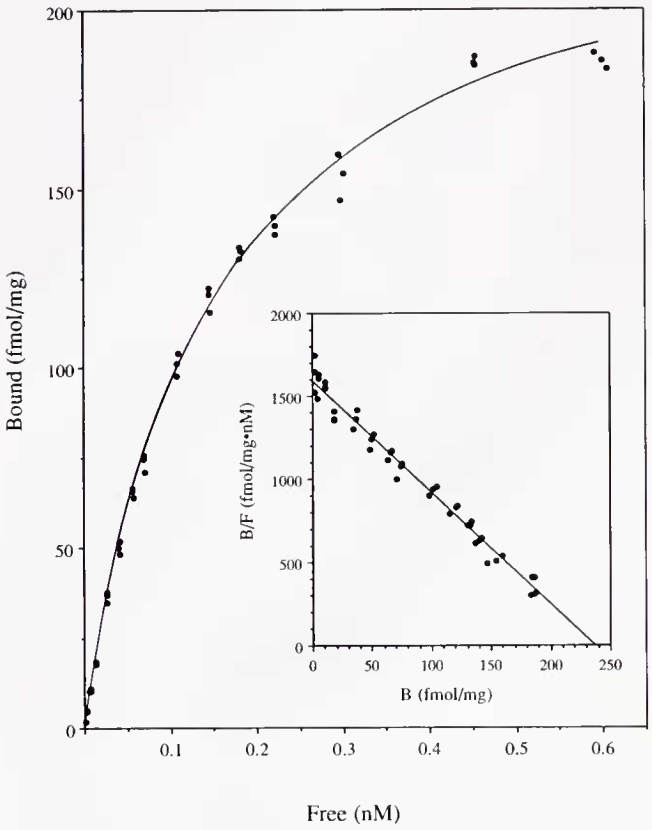


Figure 4. Saturation of specific [^{125}I]-daYFnLRFa binding to FMRFamide receptors. The specific binding of [^{125}I]-daYFnLRFa (0.002 to 0.7 n*M*) to SQM (58 μg protein) was determined after incubation for 2.5 h at 0°C. The specific binding values (fmole/mg protein) have been plotted against free radioligand concentration. Inset: Scatchard analysis of the specific binding data (r^2 = 0.98). Points are individual determinations from one experiment.

gamma counter, and specific binding (SB) was defined as the difference between total and nonspecific binding. The SB accounted for 68–82% of the total binding. The IC_{50} values for peptides were from plots of percent SB *versus* log [molar peptide]. Hill coefficients (n_H) were calculated from plots of log[%SB/(100−%SB)] *versus* log [molar peptide].

Binding of [^{125}I]-daYFnLRFa to solubilized SQM

Squid optic lobe membranes were incubated for 30 min on ice with various concentrations of CHAPS and then centrifuged at 43,000 rpm (100,000 × *g*) for 30 min. The standard protocol used in most of the experiments was 0.25% CHAPS and 1 mg/ml SQM protein. The supernatant was assayed for ligand binding activity in 40 m*M* Pipes-Tris (pH 7.0), 0.1% BSA, 1 m*M* DTT, 1 m*M* EDTA containing 10–50 p*M* [^{125}I]-daYFnLRFa. Samples (1–10 μg squid protein) were incubated in a final volume of 0.3 ml for 1.5–2.0 h at 23°C in the absence or presence of 10 μ*M* acFnLRFa. Then 0.75 ml of ice-cold 0.75% charcoal, 0.075% dextran, 20 m*M* Mes-Tris (pH 6.5), 0.02% NaN_3 was added, and the samples were mixed thoroughly. They were incubated on ice for 10–15 min to allow charcoal adsorption of free radioligand and were then centrifuged in a refrigerated microfuge (12,000 × *g*) for 2.5 min. Radioactivity in a 0.9 ml aliquot of the supernatant was measured. Under these conditions more than 95% of the unbound radioactivity was adsorbed to charcoal. Tracer integrity was determined by HPLC as before (Payza, 1987); degradation was less than 5%. Specific binding, assayed in triplicate, was linear with the amount of protein and was 5–10% of the total radioactivity added. The buffers MES, HEPES, and PIPES were equally effective in the charcoal solution.

Adenylate cyclase assays

Squid optic lobe membranes (5 μg protein) were combined with various test peptides in 300 or 400 μl of 50 m*M* Tris, 20 m*M* NaCl, 3 m*M* magnesium acetate, 500 μ*M* ATP, 1 m*M* IBMX, 50 μ*M* GTP, 20 m*M* creatine phosphate, 10 U creatine kinase, 100 μ*M* bestatin, 100 μ*M* PMSF, 15 μ*M* aprotionin, 1% BSA, pH 7.7. The pH 7.6 was chosen because the basal and acFnLRFa-stimulated cyclase were decreased by reductions of the assay pH towards the optimal binding pH of 6.5. The mixtures were incubated at 30°C for 10 min and the reactions terminated by boiling. The cAMP generated was acetylated and measured by RIA, in which an [^{125}I]-Tyr-cAMP analog was used with antiserum to cAMP (gift of Dr. David C. Klein, NICHD).

Identification of FMRFamide-like peptides in squid

Seven optic lobes (1.6 g) were homogenized in 40 ml of cold 1 *M* acetic acid with 0.02 *M* HCl. The homogenate was centrifuged at 48,000 × *g* for 10 min at 4°C, raised to pH 2.5, and applied to a C_{18} Sep-pak (Waters). The retained material was washed with 30 ml water and then eluted with 6 ml of 75% acetonitrile in 0.1% TFA (HPLC solvent B). The eluant was dried, resuspended in 2 ml methanol, and spun in a microfuge. The supernatant was dried and resuspended in 1 ml 0.1% TFA (HPLC solvent A). An aliquot (170 μl) was brought up to 15% B, microfuged again, and the supernatant was injected onto a C_4 HPLC column (Biorad) with a gradient of 15 to 30% B in 20 min at a flow of 1.5 ml/min. Aliquots of the 30-s fractions (some of which were pooled) were diluted 50-fold in 15 m*M* Pipes-Tris, pH 6.4, 0.3% BSA. In the radioimmunoassay (RIA), duplicate aliquots (20 μl) were combined with 300 μl of S253 antiserum (Price, 1983) at 1:20,000 final dilution, and 16,000 dpm of [^{125}I]-daYFnLRFa (0.01 n*M*). After incubation for 17 h at 4°C, each tube was vortexed with 1 ml cold ethanol and cen-

Table I

Inhibition of [^{125}I]-daYFnLRFa binding to SQM by FMRFamide analogs

Phe[1]	% INH	Met[2]	% INH	Arg[3]	% INH	Phe[4]	% INH
YMRFa	88.09	FIRFa	97.34	FMHFa	43.41	FMRMa	56.62
HMRFa	28.79	FFRFa	89.45	FMSFa	31.16	FMRIa	19.87
MMRFa	19.41	FLRFa	89.20	FMNFa	22.11	FMRVa	10.46
CMRFa	18.85	FVRFa	86.37	FMWFa	21.37	FMRHa	10.19
AMRFa	15.99	FYRFa	74.29	FMAFa	11.60	FMRAa	9.06
PMRFa	14.37	FWRFa	72.33	FMEFa	9.34	FMRTa	7.57
KMRFa	12.67	FCRFa	65.05	FMFFa	6.31	FMRDa	7.07
LMRFa	10.37	FTRFa	59.67	FMKFa	4.64	FMRLa	6.61
VMRFa	9.81	FHRFa	54.06	FMYFa	3.95	FMRPa	4.75
NMRFa	9.11	FQRFa	45.71	FMPFa	2.37	FMREa	3.68
EMRFa	9.09	FERFa	40.06	FMTFa	1.80	FMRKa	2.61
SMRFa	7.10	FPRFa	25.75	FMIFa	1.20	FMRYa	2.01
DMRFa	5.98	FDRFa	21.53	FMLFa	0.00	FMRRa	1.31
QMRFa	4.62	FRRFa	18.74	FMGFa	0.00	FMRNa	0.64
TMRFa	2.16	FSRFa	17.04	FMDFa	0.00	FMRQa	0.00
RMRFa	0.00	FGRFa	15.20	FMMFa	0.00	FMRGa	0.00
		FNRFa	12.74				
		FKRFa	11.04				

The specific binding of [^{125}I]-daYFnLRFa (0.05–0.06 n*M*) to SQM (15–50 μg protein) was determined in the presence of a high concentration (0.2 μ*M*, 500 times the IC_{50} of FMRFamide) of test peptide after incubation for 2.5 h at 0°C. The percent inhibition of control specific binding (mean, $n = 2$ or 3) has been displayed for each FMRFamide analog. Values below 50% indicate that the test peptide is more than 500-fold less potent than FMRFamide.

trifuged for 25 min at 4,800 × *g*. The supernatants were decanted and the radioligand binding to antiserum precipitated in the pellets was determined. The nonspecific binding was determined in the presence of 60 pmole FMRFamide; control specific binding (4980 dpm) accounted for 91% of the total binding. The inhibition of

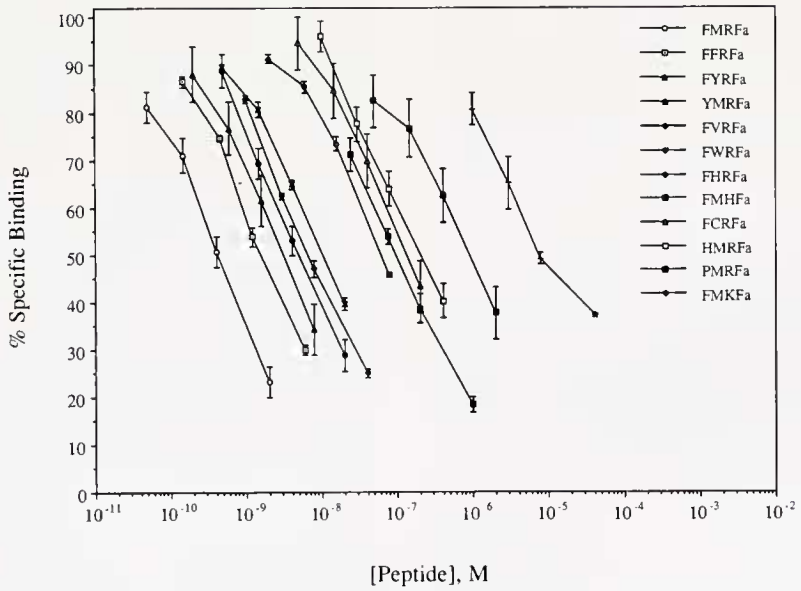


Figure 5. Structure-activity relations of peptide binding to FMRFamide receptors. The specific binding of 0.03 n*M* [^{125}I]-daYFnLRFa to SQM (30 μg protein) was determined after incubation for 2.5 h at 0°C in the presence of competing peptides at the concentrations indicated. The percent of control specific binding (mean ± SEM) has been plotted against peptide concentration. Results are from triplicate determinations of one experiment.

Table II

Potencies of peptides at membrane-bound and solubilized FMRFamide receptors

Peptide	IC_{50} (membrane)	IC_{50} (solubilized)
FMRFa	0.40	0.13
FLRFa	0.40	0.33
acFnLRFa	0.51	0.43
YGGFMRFa	1.0	0.50
FFRFa	1.4	0.55
FYRFa	2.8	0.60
YMRFa	5.0	1.7
FVRFa	6.2	3.0
FWRFa	10	2.0
FHRFa	55	6.5
FMHFa	85	100
FCRFa	120	25
HMRFa	200	50
FMKFa	7000	3000

Potencies of peptides (n*M*) in competing for [^{125}I]-daYFnLRFa binding to intact and CHAPS-solubilized SQM (with 0.03 and 0.06 n*M* radioligand respectively) were determined from displacement curves as described in the Methods.

specific binding by each sample was calculated and compared to a standard curve of FMRFamide (IC_{50} = 0.081 pmole). In the radioreceptor assay, duplicate aliquots (25 μl) were combined with 20 μg of SQM protein and 50,000 dpm [^{125}I]-daYFnLRFa (0.03 n*M*) in 300 μl of receptor binding buffer. Radioligand binding was determined after incubation for 1 h at 25°C; control-specific binding (5224 dpm) accounted for 82% of the total binding. The inhibition of specific binding was calculated, compared to a standard curve of FMRFamide (IC_{50} = 0.077 pmole), and the receptor-reactivity was plotted against elution time. The elution profiles of the FMRFamide immunoreactivty and receptor-reactivity were compared to the elution times of FMRFamide and FLRFa standards.

Identification of a FMRFamide precursor fragment in squid genomic DNA

A squid optic lobe was heated to 95°C for 10 min in 0.1 *M* NaOH. The mixture was spun in an Eppendorf centrifuge at 14,000 rpm, and 0.3 μl of the supernatant was used for PCR. The reaction mixture of 100 μl contained 200 μ*M* dNTPs, 4 U of Taq polymerase (Promega), and about 200 pmol of each primer:

Sense primer:

A C C

GAI AAG CGI TTC TTG AGG TTC GG

Anti-sense primer:

A T C A T

CAT GAA ICG TTT ICC GAA ICG CAT

The reaction mixture was covered with mineral oil and then subjected to 40 cycles of PCR under the following conditions: 94°C, 1 min; 50°C, 1 min; 72°C, 1 min. The PCR products were then cloned and sequenced.

Results

Identification of FMRFamide receptors in squid optic lobe

The presence of FMRFamide receptors in squid was examined by testing optic lobe membranes for specific binding of the FMRFamide receptor ligand [^{125}I]-daYFnLRFa. Incubation of SQM with [^{125}I]-daYFnLRFa resulted in a time-dependent increase in specific binding that attained equilibrium in 2.5 h at 0°C or 1 h at 25°C (Fig. 1). The degradation of radioligand at both temperatures was less than 10% as assayed by HPLC. The specific binding was maximal at pH 6–6.5 (Fig. 2) and was linearly dependent on the amount of membrane protein in the assay. The binding was specific for FMRFamide-related peptides (Fig. 3) and showed specificity for the C-terminal amide, since FMRF-OH (IC_{50} = 7 μ*M*) was over four orders of magnitude weaker than FMRFamide (IC_{50}

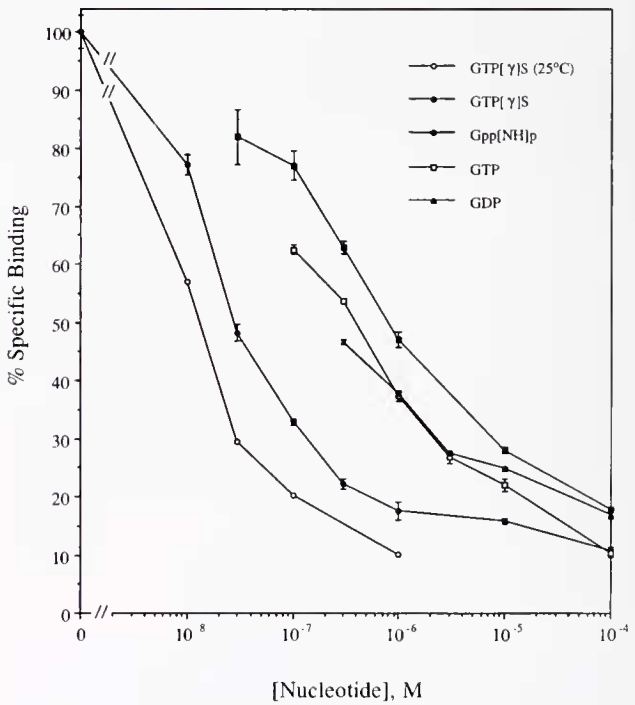


Figure 6. Nucleotide effects on FMRFamide receptor binding. The specific binding of [^{125}I]-daYFnLRFa (0.06 n*M*) to SQM (20 μg protein) was determined after incubation in triplicate for 2.5 h at 0°C in the presence of nucleotides at the concentrations indicated. An identical experiment was carried out in duplicate at 25°C for 1 h with GTP[γ]S. The percent of control specific binding (mean ± SEM) has been plotted against nucleotide concentration.

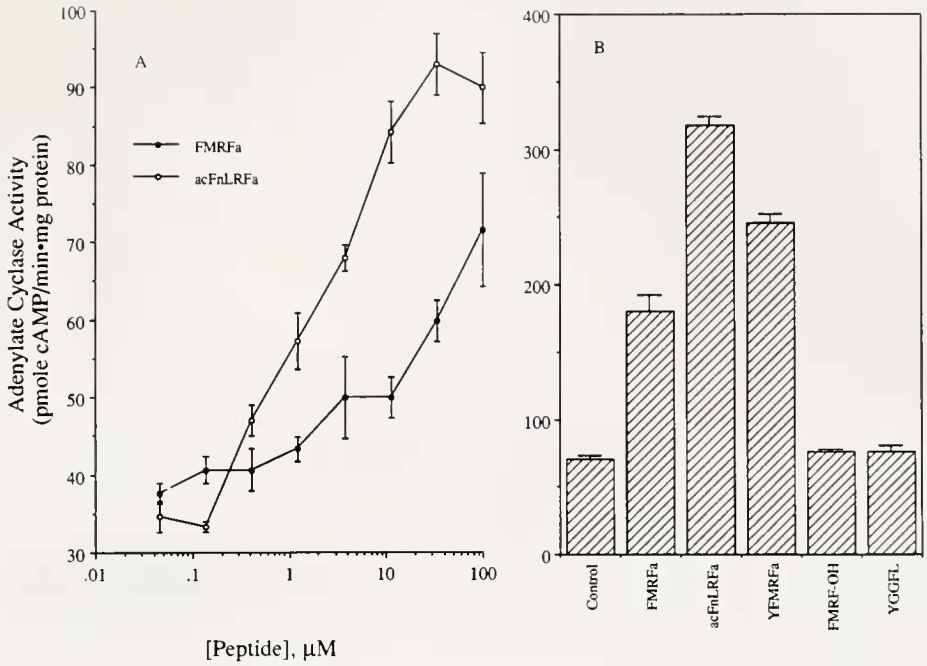


Figure 7. Stimulation of adenylate cyclase by FMRFamide-related peptides. The adenylate cyclase activity in SQM was measured in the presence of 0–100 μM peptides. (A) Dose-response curve for stimulation of adenylate cyclase by FMRFamide and acFnLRFa. (B) Effects on adenylate cyclase of peptides at 100 μM. Results are means (± SEM) of triplicate determinations from single experiments.

= 0.4 nM). The potency of FMRFamide was close to the 0.2 nM IC_{50} of the most potent ligand, daYFnLRFa (Fig. 3). The binding was not displaced by the unrelated peptides cholecystokinin octapeptide and leucine-enkephalin. Scatchard analysis of [^{125}I]-daYFnLRFa binding revealed a single population of high-affinity binding sites, with a K_d of 0.15 nM and B_{max} of 237 fmole/mg protein (Fig. 4).

Characterization of FMRFamide receptors in squid

The structure-activity relations of FMRFamide receptor binding were examined by testing a series of FMRFamide analogs for displacement of specific [^{125}I]-daYFnLRFa binding to SQM. We first examined the stereospecificity of binding, and found that D-amino acid substitution drastically reduced the subnanomolar potency of FMRFamide: [D-F]MRFa had an IC_{50} of 0.13 μM, F[D-M]RFa and FM[D-R]Fa both had IC_{50} values of 1.3 μM, and FMR[D-F]a displaced only 28% of specific binding at the highest concentration tested (4 μM). We next screened a series of analogs, L-substituted at each of the four amino acid positions, and all at 0.2 μM, to determine whether any other structures can bind to FMRFamide receptors at a concentration 500 times the IC_{50} of FMRFamide (Table I). The potencies for a selection of these FMRFamide analogs were then determined by generating displacement curves (Fig. 5) and calculating IC_{50} values for each peptide (Table II). The Hill coefficients were determined for each peptide in Figure 5, and the values ranged from 0.65 to 0.92. We also found that a FMRFamide analog with L-1,2,3,4 tetrahydroisoquinoline-3-carboxylic acid (TIC) in place of the C-terminal Phe, daYFnLR[TIC]-amide displaced radioligand binding with a potent IC_{50} of 0.39 nM (n_H = 0.87).

Coupling of FMRFamide receptors to G-proteins and adenylate cyclase

The coupling of FMRFamide receptors to G-proteins was examined by testing guanine nucleotides for inhibitory effects on specific [^{125}I]-daYFnLRFa binding to SQM. The nucleotides GTP, Gpp[NH]p, GTP[γ]S, and GDP inhibited FMRFamide receptor binding in a dose-dependent manner (Fig. 6). This effect was not observed with GMP or ATP. The inhibitory effect of GTP[γ]S at 25°C was about 2.5-fold more potent than at 0°C (Fig. 6), a finding consistent with a previous report (Aronstam and Narayanan, 1988). The coupling of FMRFamide receptors to adenylate cyclase was examined by testing FMRFamide for modulation of the enzyme activity in SQM. Production of cAMP from ATP by adenylate cyclase in SQM was stimulated in a dose-dependent manner by FMRFamide (Fig. 7A). The peptides acFnLRFa and

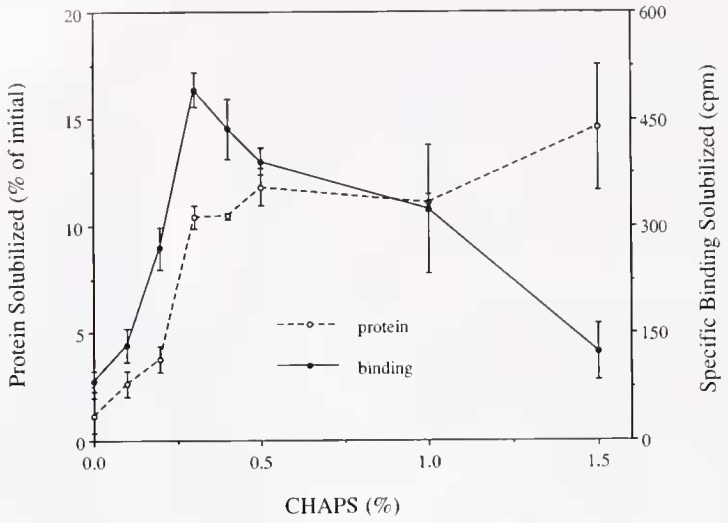


Figure 8. Solubilization of FMRFamide receptors from SQM. Membranes (1 mg/ml) were incubated with CHAPS on ice for 30 min, centrifuged at 100,000 × *g* for 30 min, and the supernatant was assayed for protein and specific binding in the presence of ~0.05 n*M* [^{125}I]-daYFnLRFa. Similar results were obtained in four independent experiments and at initial protein concentrations of 2, 5, and 10 mg/ml.

YFMRFa were more active than FMRFa, but FMRF-OH and Leu-enkephalin were without effect (Fig. 7B). If a G-protein participates in the activity of FMRFamide-stimulated adenylate cyclase, then GTP must be required, and this requirement was examined. In the presence of GTP (20 or 50 μM), 100 μM acFnLRFa produced a two-fold stimulation of adenylate cyclase, but in the absence of GTP it had no effect.

Solubilization of FMRFamide receptors

In exploratory studies, the following observations were made. Specific [^{125}I]-daYFnLRFa binding activity could be solubilized with digitonin (0.25–1.0%) over a broader range of detergent concentrations than with CHAPS, but the recoveries were not as high as those achieved with CHAPS. CHAPSO at 0.25% also solubilized specific binding activity. CHAPS solubilization was optimal at pH 6.5, whereas solubilized ligand binding activity was optimal at pH 7.0. Solubilization of ligand binding activity was reduced at high ionic strength (50% less in 0.15 *M* NaCl or KCl). The measurement of binding activity was much less sensitive to ionic strength but was significantly reduced by divalent cations (40% less in 5 m*M* $MgCl_2$ or $CaCl_2$). The CHAPS-solubilized preparation was stable for at least 6 h on ice, and stability was not affected by sucrose or glycerol. Protease inhibitors (PMSF, benzamidine, bestatin, bacitracin, leupeptin, aprotinin, pepstatin) did not consistently improve the recovery of binding activity when they were added either during the solubilization or during the binding incubation. Finally, both [^{125}I]-D-YFnLRFa and [^{125}I]-YGGFLRFa bound to the solubilized preparation with similarly high affinity and specificity but with more nonspecific binding as compared to [^{125}I]-daYFnLRFa.

In the experiments reported below and illustrated in the figures, the zwitterionic detergent CHAPS was used to solubilize the FMRFamide receptor from SQM. CHAPS concentrations in the neighborhood of the critical micelle concentration (about 0.4%) solubilized 10% of the total membrane protein (Fig. 8). The optimal solubilization of specific binding activity occurred in the range 0.25–0.5% CHAPS. The decrease of binding activity recovered at higher CHAPS concentrations probably was due to denaturation of the receptor as a consequence of its direct interaction with detergent or of delipidation. Alternatively, higher CHAPS concentrations might disrupt the association of the receptor and G-protein, causing a loss in receptor binding affinity.

Characterization of solubilized FMRFamide receptors

The time courses of association of radioligand with receptor and of dissociation of radioligand from receptor after addition of 1 μM unlabeled ligand are shown in Figure 9. Association under pseudo first-order conditions reached a plateau after 90–120 min incubation at room temperature; a semilogarithmic plot of the approach to equilibrium as a function of time (inset) yielded $k_{on} = 9.6 \times 10^7\ M^{-1}\ min^{-1}$. Ligand dissociation was slow and yielded $k_{off} = 0.00894\ min^{-1}$ for an equilibrium dissociation constant of 0.093 n*M*, which was slightly more potent (1.6-fold) than the K_d measured in unsolubilized SQM (0.15 n*M*). Scatchard analysis of specific binding as a

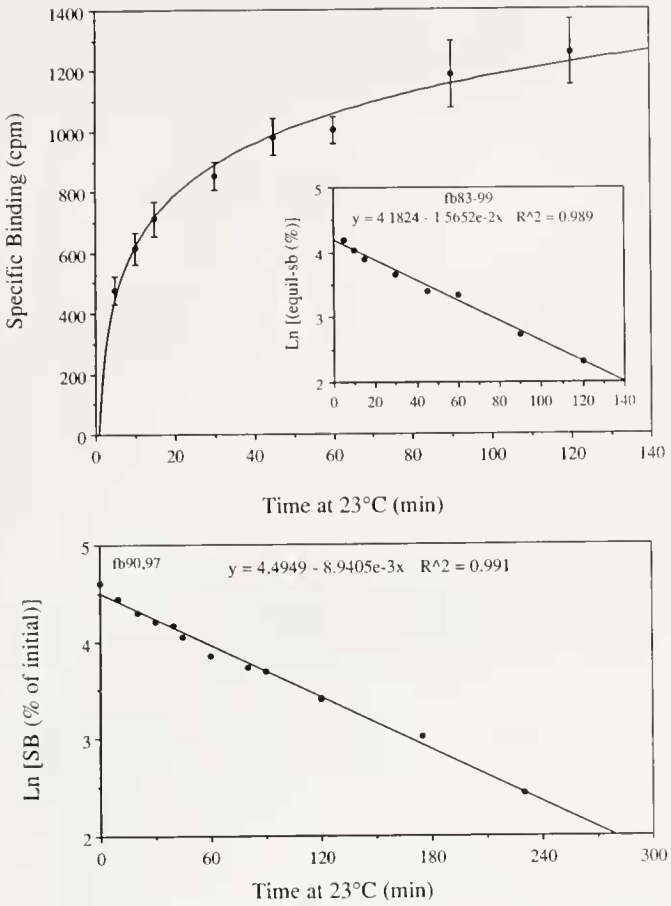


Figure 9. Association and dissociation of receptor and ligand. (A) Solubilized SQM was combined with 0.07 nM [^{125}I]-daYFnLRFa and assayed for specific binding as a function of time. The equilibrium value was estimated to be 1400 cpm from five independent experiments. The inset shows the relation of the difference between the actual and equilibrium values of binding as a function of time, under pseudo first-order conditions with [ligand]/[receptor] ~7. (B) Solubilized SQM was incubated with radioligand for 3 h, and then 1 μM acFnLRFamide was added. The amount of bound radioactivity remaining was determined as a function of time in two independent experiments. The initial value was taken as the amount bound just prior to addition of acFnLRFamide.

function of ligand concentration produced curved plots whether CHAPS or CHAPSO was employed to solubilize the receptors (Fig. 10). The curved Scatchard plots made it difficult to estimate precisely the affinity and yield of receptors from the solubilization step. Using a nonlinear curve-fitting program (JMP, SAS Institute, Cary, NC), we determined that the data could not be fit to a two-site model.

Binding to the solubilized receptors was sensitive to guanine nucleotides as for the membrane-bound receptors (Fig. 11). Again, GTP[γ]S was more potent than GTP, and ATP had no effect over the same range of concentrations. The IC_{50}s for GTP and GTP[γ]S were 0.17 μM and 0.028 μM, each about 200-fold more potent than observed with unsolubilized SQM. The complete inhibition by GTP and GTP[γ]S indicated that all of the active, solubilized receptors were complexed with G-proteins. These results are consistent with a GTP-induced decrease in the affinity of the receptor for ligand, because the charcoal adsorption assay does not detect interactions with $K_d > \sim 0.1$ μM. Scatchard analysis in the presence of 0.17 μM GTP produced a curve, parallel to that shown in Figure 10, but shifted downward with an apparent loss of about half of the binding sites (not shown).

The binding specificity of the solubilized receptors was determined by displacement of the radioligand with other peptides. As shown in Figure 12, FMRFamide and acFnLRFa both had IC_{50}s < 1 nM, whereas the non-amidated FMRF had an IC_{50} of 30 μM. Selected FMRFamide analogs (from a panel of 60 peptides) displayed intermediate IC_{50}s as shown in Table II, whereas the unrelated peptides leucine-enkephalin, methionine-enkephalinamide, somatostatin, and substance P (7–11) did not displace at concentrations up to 10 μM. The competition curves for displacement of radioligand from the solubilized receptor were similar in maximal displacement and steepness for many of the FMRFamide analogs tested, suggesting displacement from a single population of sites. Hill plots yielded coefficients of 0.52–0.86, indicating noninteracting binding sites. The log[IC_{50}] values determined for each peptide with the CHAPS-solubilized receptors

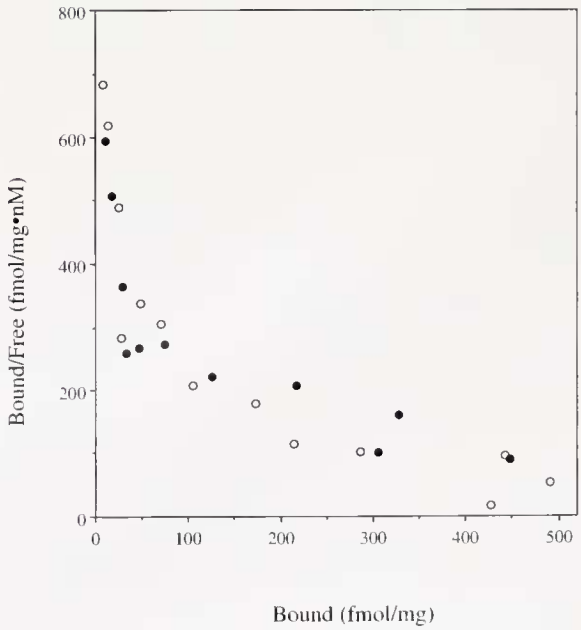


Figure 10. Scatchard plot of binding to solubilized receptors. The specific binding of increasing concentrations of [^{125}I]-daYFnLRFa to solubilized SQM was determined. The values of bound/free radioligand (fmole/mg protein • nM) have been plotted against bound radioligand. Results are from single experiments in which the receptors were solubilized with CHAPS (closed circles) or CHAPSO (open circles).

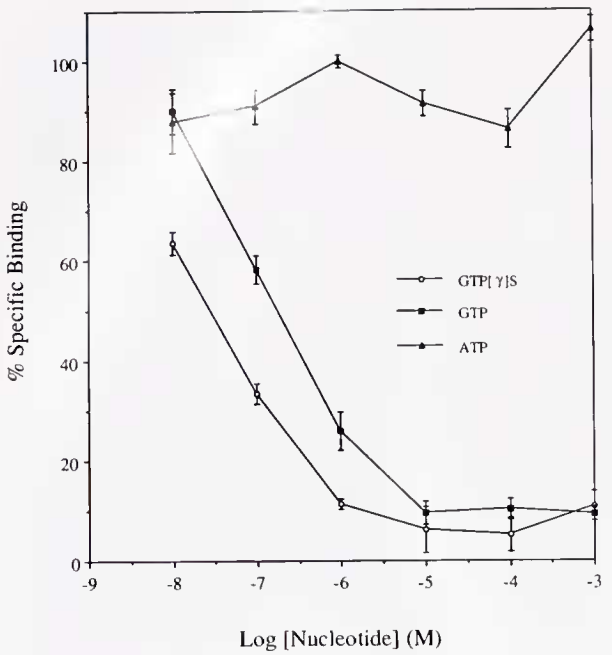


Figure 11. Nucleotide effects on solubilized receptors. The specific binding of 0.07 n*M* radioligand to solubilized SQM was determined in two independent experiments with the nucleotides GTP, GTP[γ]S and ATP. The percentage of control specific binding (mean ± SEM) has been plotted against nucleotide concentration.

were linearly related to those determined with the membrane-bound receptors, and the slope was unity (Fig. 13). This finding suggests that the binding specificities of the two preparations were nearly identical, and that CHAPS treatment had not altered the structure of the protein. Overall, solubilization improved displacement potency of the peptides tested by an average of half a log unit (threefold).

Identification of FMRFamide-like peptides in squid optic lobe

The demonstration of FMRFamide receptors in squid optic lobe suggests that FMRFamide itself should be detectable in this tissue. An acid extract of squid optic lobes was subjected to reverse-phase HPLC, and the fractionated eluant was analyzed both by radioimmunoassay (RIA) and by the radioreceptor assay described above. Some of the fractions were immunoreactive in the RIA with [^{125}I]-daYFnLRFa and anti-YGGFMRFamide antiserum S253 (Price, 1983). Most of the FMRFamide-immunoreactivity eluted in the positions of authentic FMRFamide and FLRFa (Fig. 14A). Moreover, the same HPLC fractions of the extract displaced [^{125}I]-daYFnLRFa from binding sites in squid optic lobe membranes; thus, the receptor-reactivity coeluted with the immunoreactivity in the positions of FMRFamide and FLRFa (Fig. 14B). The results of both assays were in close agreement that the amount of FMRFamide in the tissue (5.2–6.4 nmole/g wet weight) was about 13- to 14-fold that of FLRFa (0.36–0.51 nmole/g). An acetone extract of squid optic lobes was also subjected to reverse-phase HPLC on a C_{18} column, and the fractions were assayed with S253 antiserum and [^{125}I]-YGGFMRFamide. Again, some of the immunoreactivity

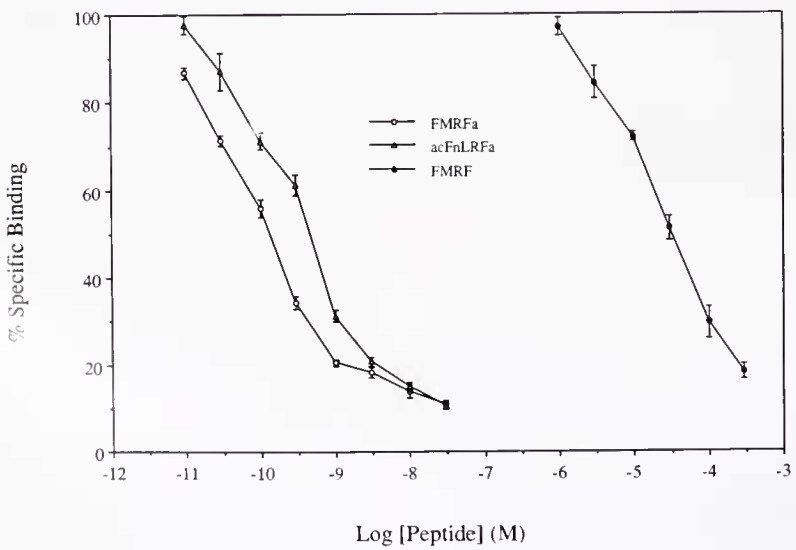


Figure 12. Binding specificity of solubilized receptors. The specific binding of 0.07 n*M* radioligand to solubilized SQM was determined in the presence of FMRFamide, acFnLRFamide, and the non-amidated FMRF at the indicated concentrations in many independent experiments. The percentage of control specific binding (mean ± SEM) has been plotted against peptide concentration.

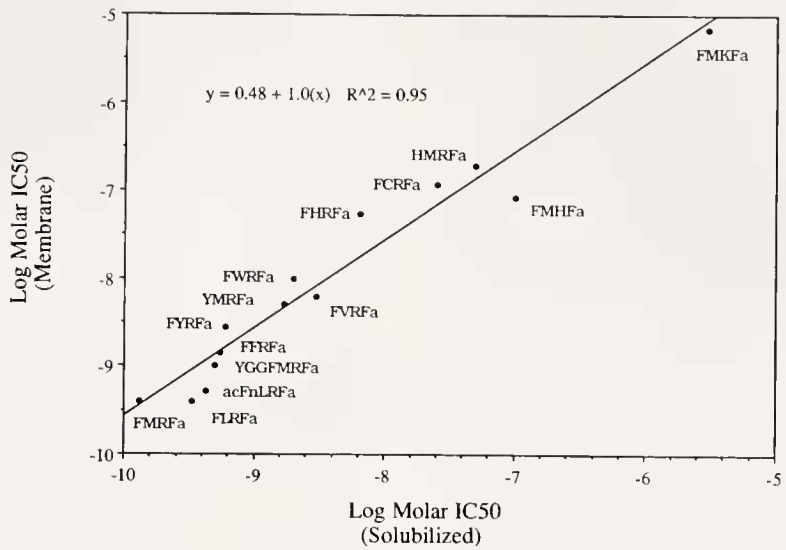


Figure 13. Comparison of the CHAPS-solubilized and membrane-bound FMRFamide receptors. FMRFamide analogs were tested for their ability to displace radioligand binding from CHAPS-solubilized and unsolubilized SQM, and their log (IC_{50}) values (from Table II) were plotted together. Results are compiled from many separate experiments.

eluted in the positions of authentic FMRFamide and FLRFa, and unidentified, later-eluting peaks were also detected by S253 antiserum (not shown).

Identification of a FMRFamide precursor fragment in squid genomic DNA

As demonstrated above, coelution in distinct parallel assays strongly indicates that FMRFamide and FLRFamide are present in squid optic lobes. Nevertheless, in the absence of information from either sequencing or mass spectrometry, these data are not unimpeachable. Moreover, although FMRFamide and FLRFamide have been chemically identified in every major class of molluscs (Price and Greenberg, 1989), including cephalopods (Martin and Voigt, 1987), this has never been done in any squid. Still, rather than identifying these particular peptides in yet another species of mollusc, we proceeded to clone and sequence part of a FMRFamide precursor from squid genomic DNA. Following the strategy described in the Methods, a fragment of the genome was amplified by PCR, then cloned and sequenced (Fig. 14C). This fragment encodes, between the primers, three copies of the peptide: Lys-Arg-Phe-Met-Arg-Phe-Gly-Arg, where the basic residues at the *C*- and *N*-terminals are cleavage sites, and the glycyl residue is the amidation site.

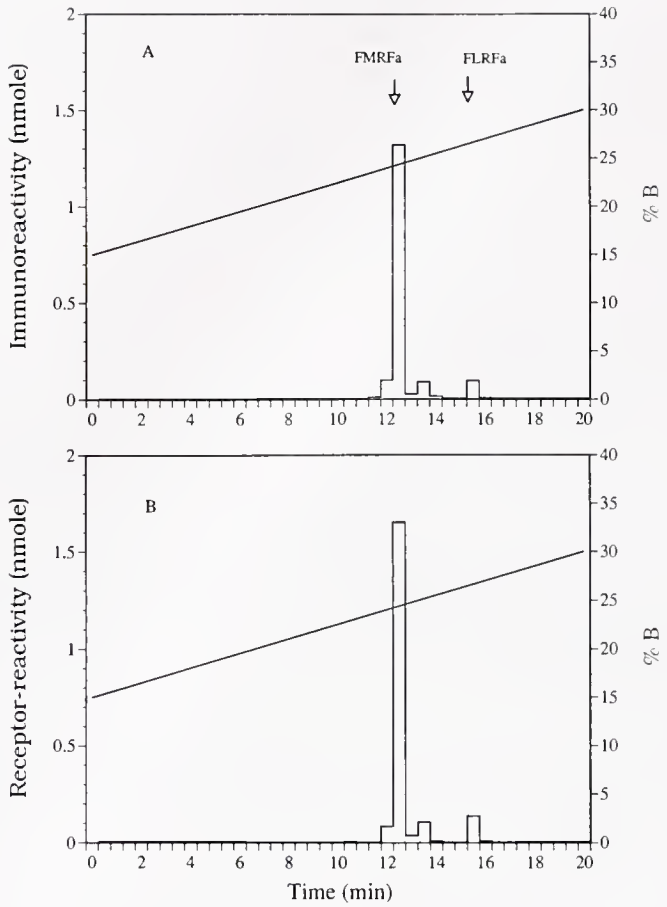
Discussion

FMRFamide receptors in squid

Our finding of specific, saturable [^{125}I]-daYFnLRFa binding to optic lobe membranes of *Loligo pealei* constitutes the first direct evidence of FMRFamide receptors in this tissue. The ability of [^{125}I]-daYFnLRFa to specifically label FMRFamide receptors in *Loligo* is consistent with the prior use of this radioligand in the land snail *Helix aspersa*, the main similarity between the receptors of the two molluscs being the low binding potency of FMRF-OH with respect to FMRFamide (Payza, 1987). These strict preferences for the amidated form of the peptide are consistent with the bioactivity of the peptides on the isolated *Helix* heart (Payza, 1987). Similarly, FMRFa, FLRFa, YGGFMRFa, and YGGFLRFa were cardioexcitatory on the isolated systemic heart of *Octopus vulgaris*, and these effects were specific for the *C*-terminal amide (Martin and Voigt, 1987; Voigt *et al.*, 1981). The main differences between the receptors of *Helix* and *Loligo* are that (1) in squid [^{125}I]-daYFnLRFa binds to a single population of binding sites, whereas in *Helix*, multiple sites were observed (Payza, 1987; Payza *et al.*, 1989); (2) the receptors in squid show a much higher affinity for FMRFamide; and (3) *N*-terminal extensions greatly improved the micromolar potency of FMRFamide at *Helix* receptors, but did not improve the already subnanomolar FMRFamide affinity at squid receptors; *i.e.*, the potencies of acFnLRFa and daYFnLRFa were similar to that of FMRFamide, and YGGFMRFa was 2.5-fold weaker. The pH optimum of binding was also more acidic in squid than in *Helix*.

Structure-activity relations of receptor binding

In contrast to the minor effects of the *N*-terminal extensions, the effects of single amino acid substitutions in



C

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GAG AAG AGG TTC TTG AGG TTC GGT AAG TCA GAG GAC AAA AGG TTC   45
E   K   R   F   L   R   F   G   K   S   E   D   K   R   F

ATG AGA TTT GGC CGA GAC CCC AGC GAT GTT GAA GAT GAA TTG GAA   90
M   R   F   G   R   D   P   S   D   V   E   D   E   L   E

GAG GAC AAA CGT TTT ATG CGA TTC GGA CGC GGC GCC GAA GAT GAT  135
E   D   K   R   F   M   R   F   G   R   G   A   E   D   D

GAG GAA GAA GCC GAG AAG AGA TTT ATG AGA TTT GGA CGT GAC CCC  180
E   E   E   A   E   K   R   F   M   R   F   G   R   D   P

GAA AAG AAA TTC ATG CGC TTC GGC AAA CGC TTC ATG              216
E   K   K   F   M   R   F   G   K   R   F   M
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FMRFamide ranged from no effect to the complete elimination of binding at 0.2 μM peptide. In the Phe[1] position, the least detrimental substitution was with Tyr; all other substitutions tested were not tolerated (*e.g.*, HMRFa and PMRFa had IC_{50}s of 0.2 and 1.0 μM, respectively). The Met[2] was the position most tolerant to substitution; FLRFa was equipotent with FMRFamide, and analogs containing aromatic residues were also active (see FFRFa, FYRFa, FWRFa). The Arg[3] could not be substituted with the positively charged Lys, but it could be replaced by His with a 200-fold loss in binding. The *C*-terminal Phe was extremely intolerant to substitution with almost any amino acid tested; the only exception was the rigid Phe analog, TIC. The potent binding of daYFnLR[TIC]amide suggests it should be a potent agonist or antagonist.

FMRFamide receptor coupling

Our results suggest that FMRFamide receptors in squid optic lobe are coupled to G_s. The specificity of the guanine nucleotide effect on FMRFamide receptor binding is consistent with GTP regulation of other G-protein-coupled receptors, such as receptors for opiates (Childers and Snyder, 1980) and the mammalian FMRFamide-related peptide Neuropeptide FF (Payza and Yang, 1993). Moreover, the stimulation of adenylate cyclase by FMRFamide in squid shows a requirement for GTP. Thus, squid FMRFa receptors appear to be members of the family of G-protein-coupled receptors, one of which (phospholipase *C*-coupled rhodopsin) has been cloned recently in squid (Hall *et al.*, 1991). The coupling of FMRFamide receptors in optic lobe of *Loligo* is in agreement with FMRFamide-stimulated cAMP production in *Mercenaria* heart (Higgins *et al.*, 1978), *Lampsilis* heart (Painter, 1983), and *Aplysia* gill (Weiss *et al.*, 1984). The low potency of FMRFamide receptor-stimulated adenylate cyclase in SQM is consistent with the observed inhibitory effect of GTP on receptor binding. The larger effects of acFnLRFa and YFMRFa relative to FMRFamide suggest that the *N*-terminally modified peptides may have higher efficacies at the receptor.

Solubilized FMRFamide receptors

The FMRFamide receptor in the squid shares with the family of G-protein-coupled receptors the characteristic that it can be solubilized by digitonin, CHAPS, and CHAPSO (all detergents containing the steroid skeleton), while still retaining the ability to bind ligands (Haga *et al.*, 1990). This family of receptors consists of integral membrane proteins of 40–50 kDa, with seven membrane-spanning regions and a ligand-binding pocket buried within the transmembrane domain of the protein (Dohlman *et al.*, 1987; Lismaa and Shine, 1992). In some instances, as with the FMRFamide receptor, solubilized receptors retain their association with the heterotrimeric G-proteins and display GTP-sensitive ligand affinity as in the intact cell (Berrie *et al.*, 1984; Knuhtsen *et al.*, 1988; Marie *et al.*, 1989; Gimpl *et al.*, 1990). The immunoprecipitation of somatostatin receptors has been reported with antibodies directed against G-proteins (He *et al.*, 1990; Law *et al.*, 1991), and this may provide a useful purification step for use with the FMRFamide receptor. In addition, the increased potency of guanine nucleotides observed in the solubilized preparation is consistent with recent reports of similar changes in the neuropeptide Y and somatostatin receptors (Marie *et al.*, 1989; Gimpl *et al.*, 1990).

The unsolubilized FMRFamide receptors appeared to be a single population of high affinity, non-interacting sites. After CHAPS-solubilization, the kinetic analysis of radioligand binding and the potencies of FMRFamide analogs in the displacement experiments suggest that high affinity binding is slightly enhanced by CHAPS treatment. The unchanged structure-activity relations of peptide binding suggest that the detergent did not adversely affect the ligand binding site, but the curvilinear Scatchard plot does indicate some heterogeneity. Considering also the Hill coefficients of 0.5–0.9 for the peptide analogs, we suggest that the solubilized preparation contains a mixture of receptors, some of which have been perturbed by detergent and show a decreased affinity for ligand. This heterogeneity would account for the nonlinear Scatchard

Figure 14. Identification of immunoreactive and receptor-reactive FMRFamide in squid optic lobe. (A) In one experiment a squid optic lobe extract was analyzed on a C_4 HPLC column as described in the Methods. The fractions were analyzed by RIA with S253 antiserum, and the FMRFamide immunoreactivity has been plotted against time. The peaks eluting in the positions of FMRFamide and FLRFa (indicated with arrows) inhibited 76 and 44% of specific binding in the assay, corresponding to 1.32 and 0.096 nmoles, respectively. (B) The fractions from the HPLC in A were analyzed by radioreceptor assay with squid optic lobe membranes and [^{125}I]-daYFnLRFa, and the FMRFamide receptor-reactivity has been plotted against time. The peaks eluting in the positions of FMRFamide and FLRFamide inhibited 82 and 54% of specific binding in the assay, corresponding to 1.65 and 0.14 nmoles, respectively. (C) A squid optic lobe was heated to 95°C for 10 min in 0.1 *M* NaOH. The mixture was spun in an Eppendorf centrifuge at 14,000 rpm, and 0.3 μl of the supernatant was used for PCR with the primers and conditions described in the Methods. The sequence of one of the cloned PCR products is shown.

plots and the Hill coefficients less than unity, and would also be consistent with the sub-n*M* IC_{50}s measured at low radioligand concentrations (conditions under which the highest affinity binding sites are primarily detected). At higher radioligand concentrations, as in the Scatchard analyses, the lower affinity sites are occupied and contribute to the observed values of specific binding. Since the membrane-bound receptor shows no sign of heterogeneity, it seems clear that solubilization is responsible for producing it. In that case, the optimal recovery of high affinity FMRFamide receptors from SQM will require the development of protective conditions to preserve high affinity binding. Once optimal conditions are achieved, the high affinity, specificity, and abundance of FMRFamide receptors in squid optic lobe suggest it is an ideal source from which to purify the receptors.

FMRFamide-like peptides in squid

We have clearly shown that FMRFamide and related peptides are present in squid optic lobe. First, HPLC peaks of FMRFamide-like reactivity that elute with synthetic FMRFamide and FLRFa have been detected both with a specific RIA and with a specific radioligand receptor assay. The concordance of these parallel assays already suggests strongly that squid optic lobes contain authentic FMRFamide and FLRFa. This conclusion is further reinforced by our detection, in genomic DNA prepared from optic lobe, of a gene encoding the FMRFamide precursor protein. Unidentified peaks of FMRFamide reactivity were also detected. One of these, eluting between FMRFamide and FLRFa, contained material that binds to both the FMRFamide antiserum and the receptor; later-eluting peaks from acetone extracts were also detected, but only by their immunoreactivity. Thus, other FMRFamide-like peptides probably occur in squid optic lobe. These findings are consistent with the notion, based on numerous observations, that FMRFamide, FLRFamide and various homologs, some *N*-terminally extended, occur in all molluscs (Price and Greenberg, 1989). More to the point, however, Martin and Voigt (1987) have identified FMRFamide, FLRFa, AFLRFa, and TFLRFa in the optic lobe of an octopodid cephalopod, *Octopus vulgaris*. Our identification of both FMRFamide-related peptides and FMRFamide receptors in squid optic lobes indicates that members of this peptide family function as neurotransmitters in this part of the central nervous system. Such a regulatory role for FMRFamide has been described for the squid giant synapse (Cottrell *et al.*, 1989), but further studies will be required to elucidate the functions of FMRFamide-like peptides in the optic lobe.

Acknowledgments

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